GLUTATHIONE-CONTROLLED ANAEROBIOSIS IN CRYPTOCERCUS, AND ITS DETECTION BY POLAROGRAPHY [1, 2]

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The flagellate protozoa of *Cryptocercus punctulatus,* hind-gut symbionts of this wood-feeding roach, are generally regarded as obligate anaerobes. Cleveland *et al.* (1934) and Cleveland and Burke (1956) reported high oxygen susceptibility for *Cryptocercus* protozoa during *in vivo* studies relating oxygen toxicity with tension and temperature changes. Closely related termite protozoa are also killed by oxygen, demonstrated *in vivo* (Cleveland, 1925a, 1925b; Yamaski, 1931) and *in vitro* (Trager, 1933; Hungate, 1939). Trager's culture experiments with termite (*Zootermopsis*) protozoa, however, indicated to him that one flagellate species, *Trichonympha sphaerica,* probably requires oxygen in a very low but definite concentration.

It has been proposed that anaerobic status, *in strictu sensu,* might be assigned hind-gut-residing termite and roach protozoa if protozoa survival accompanied host survival following prolonged exposure of the host to nitrogen. Gilmour (1940) observed flagellate death in termites exposed to nitrogen and concluded that these protozoa are killed in their own metabolic end-products rather than from oxygen lack. Unpublished studies (Ritter) using *Cryptocercus* support Gilmour's conclusion. In brief, rapid cell destruction follows organic acid accumulation and pCO_2 rise with subsequent hind-gut fluid loss.

In all of these experiments it is impossible to define the exact status of oxygen (whether applied in excess of atmospheric concentration or totally omitted) and its influence upon the protozoa. Complex and critical side effects induced by secondarily altered chemical and physical constants must be assumed. Furthermore, it has not been established that a minute trace of oxygen might not be present, adequate, and quite essential for protozoan requirements. Indeed it is difficult to visualize a hind-gut content totally anaerobic considering its confinement in tissues liberally supplied with tracheal endings.

The immediate need, before further attempts to isolate the exact oxygen effect upon the protozoa of *Cryptocercus,* was development of a medium for *in vitro* culture. Actual design of the *in vitro* culture, however, was predicated upon a more certain understanding of the oxygen concentration, if any, present in roach hind-gut content.

The following study involves an apparently successful attempt to qualify anaerobiosis with respect to degree in very small samples of *Cryptocercus* hind-gut

[1] This investigation was supported by Public Health Service Grant E-852(C3), National Institute of Allergy and Infectious Diseases, to L. R. Cleveland and H. Ritter, Jr.; (C4) and (C5) to H. Ritter, Jr.

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content with the highly oxygen-sensitive polarograph. Related phenomena are described and discussed to the extent present evidence will allow.

Materials and Methods

1. *The experimental animal*

A large collection of *Cryptocercus* was maintained at 20° C. in a metal container. Individuals dwelled within and fed upon portions of the same partially decomposed wood in which they were found. Only adult roaches were selected to supply material for the polarographic study. Hind-gut fluid was removed from the roach by holding it between thumb and forefinger, and squeezing with pressure strokes in an antero-caudad direction on the abdominal region. For a description of this insect as well as the protozoan symbionts it contains, see L. R. Cleveland *et al.*, (1934).

2. *Polarography*

Polarography, utilizing an apparatus with a sensitivity limit of approximately 10^{-6} *M* (0.001 m*M*) is one of the most efficient and accurate methods for oxygen detection (Kolthoff and Lingane, 1952). The normal hind-gut volume of an adult roach approximating only 0.1 ml., an abundance of suspended cellular and cellulose constituents in a brown and rather viscous liquid, and the likelihood of encountering a very low partial pressure of oxygen are factors which initially eliminated other techniques from consideration.

A Sargent Model XI recording polarograph was used in conjunction with a micro H-cell, possessing a solution chamber functional with slightly less than 0.1 ml. of sample. Although the micro-cell was designed and constructed with a side arm loading port suggested by Beecher, Follansbee, Murphy and Craig (1942), its use as a means of protecting the sample from air during loading proved to be unnecessary in this work. A constant flow of nitrogen was maintained while the roach hind-gut substance was squeezed into the micro-cell, during insertion of the mercury-dropping electrode, and for duration of the analysis.

Preparation of the saturated KCl-agar gel used in the salt bridge of the micro-cell and the saturated KCl solution for use in the reference electrode was performed according to Kolthoff and Lingane's method (1952). This reference and that of L. Meites (1955) offer detailed reviews of polarographic theory.

A polarogram is a plot of the current flowing through the solution in a polarographic cell against the potential of the mercury-dropping electrode. Polarograms used to document this study were traced and arranged to follow generally the order of discussion.

All polarograms were made with the same dropping electrode capillary at a constant rate of mercury drop formation. The 20° C. water bath surrounding the electrolysis vessel and all other operating conditions involved in polarographic technique were held constant.

Since the comparative method of wave height-concentration plot was used, rather than the Ilkovič equation to calculate the concentration of substance present, it is necessary to note that not all polarograms included could be recorded at the same sensitivity.

In order to record a high substance concentration, a polarograph setting was selected (*e.g.* shunt X-5 or X-10), resulting in lower apparatus sensitivity. The full wave height formed between the residual current (the lower plateau of the polarogram) and the limiting current (the upper plateau of the polarogram) wave portions, therefore, could be included within the polarographic paper margins. A low substance concentration could be recorded conveniently at a polarographic setting of shunt X-1 or X-2 while utilizing maximum apparatus sensitivity. The shunt setting, used in recording a polarogram, is included in legend data corresponding to the lettered curve figured. When a quantitative comparison is made between two polarograms, concentration reading corrections must be made for polarograms recorded at different sensitivity settings. For example, in a polarogram recorded at an apparatus shunt setting X-2, sensitivity would be one-half that utilized in a record made at a setting of X-1. Likewise, a setting X-1 indicates a recording sensitivity five times greater than a setting X-5. The wave height (corrected when necessary as directed) represents concentration, and this is read in microampere units of current. The voltage range in all polarograms extends from 0 to −0.4 volt.

3. *Glutathione assay*

The procedure used for a sodium nitroprusside assay of reduced glutathione was developed by Grunert and Phillips (1951), although in this study sodium chloride and metaphosphoric acid were not included as in Benesch, Benesch and Rogers (1953).

The alloxan (alloxan monohydrate, Eastman) "305" method of colorimetric determination, according to Patterson, Lazarow and Levey (1949), was modified only to the extent of reducing reaction volumes for adaptation to the Beckman-Spinco ultramicro analytical system. Application of the Beckman DU spectrophotometer to minute quantity measurement is described by Lowry and Bessey (1946).

The microbioassay for reduced glutathione, based on the "feeding reaction" of *Hydra* worked out by W. F. Loomis (1955a, 1955b), was performed in the Loomis Laboratory.

The polarograph, already discussed as applied, registered a decomposition voltage specific for the sulfhydryl group of a thiol compound. The comparative method of polarography identified the recorded substance to be reduced glutathione.

4. *Reagents*

Reduced glutathione as well as all other substances used in the comparative approach—polarographically to identify materials responsible for wave formation—were obtained from the Nutritional Biochemicals Corporation. Triple-distilled mercury was used in all polarographic procedures.

"Pre-purified" nitrogen (Airco) assayed 99.998% and oxygen (Airco) assayed 99.5%. Gases were passed through gas-equilibrated water and directed by capillary pipette into the micro-cell solution chamber. During routine polarographic procedure, nitrogen was passed over the surface of the gut content sample. In certain specified instances oxygen or nitrogen, as the case may have been, was bubbled through the sample *via* a capillary. The latter procedure, when applied, was carried out immediately prior to analysis.

Results

1. *Polarograms recorded immediately following sample isolation from the hind-gut*

Dissolved oxygen in solution is reduced to hydrogen peroxide at the dropping mercury electrode. Kolthoff and Lingane (1952) reported the half-wave potential of the corresponding wave, indicating this reduction is practically independent of pH, at a reading of −0.05 volt *versus* a saturated calomel electrode (S.C.E.).

More than 100 polarograms have been recorded of *Cryptocercus* hind-gut content. The samples ranged in volume from slightly less than 0.1 ml., when one individual roach was used, to approximately one ml. collected from 10–12 roaches. It was impossible to demonstrate an oxygen wave. In particular, ordinary exposure of the gut content to air, incidental to its hasty transfer from insect to the solution chamber of the polarographic cell, failed to introduce a detectable amount of oxygen (Curve A, Figs. 2, 3, 5; Curves A and B, Fig. 6).

Polarograms consistently included a prominent wave at a negative half-wave potential (−E ½), ranging between −0.19 to −0.25 v. *vs.* S.C.E. Absence of complete specificity in the −E ½ reading suggested the recorded substance within the gut content was organic and pH-dependent. According to Meites (1955), a nonspecific E ½ associated with a substance showing polarographic reversibility (as appears within gut content) is more likely attributable to a variable pH than to varying ionic strength in an unaltered medium with regard to buffer.

No direct evidence for the correlation between −E ½ and the initial gut content pH for use as a reference point was sought. It was anticipated that delay, in the event sufficient sample remained for polarographic study after pH measurement of a volume as small as 0.1 ml., would be accompanied by sample deterioration. Cell activity gradually changes the chemical nature of hind-gut content as confined metabolites accumulate. The pH of freshly obtained gut content samples ranges between 6.6 and 7.2.

Attempted sample pH control during polarography by oxygen-free buffer addition probably resulted in failure for one or more of the following reasons: (1) sudden medium alteration due to contact with lysed cell content, (2) severe sample dilution, and (3) ionic strength change leading to nonspecificity of −E ½ identical to that caused by variable pH for which control was sought ionically.

Slight variation, therefore, in −E ½ between polarograms of different freshly obtained gut content samples is attributed to pH variation between samples. Initial standardization of pH, and, for that matter, pH control during a series of recorded polarograms tracing changes produced by *aging* of any one sample as reported later, cannot be achieved successfully with buffer. Regardless, a low oxidation-reduction potential is indicated, and specificity range is sufficiently limited to allow positive identity of the reducing agent.

2. *Tests used in identification of the reducing agent*

A. Polarography

Separate polarograms for solutions of varied substances in 0.1 *M* KCl failed to produce waves even remotely resembling the shape of Curve A, Figure 2, much less a half-wave potential falling within the desired range. Among compounds studied

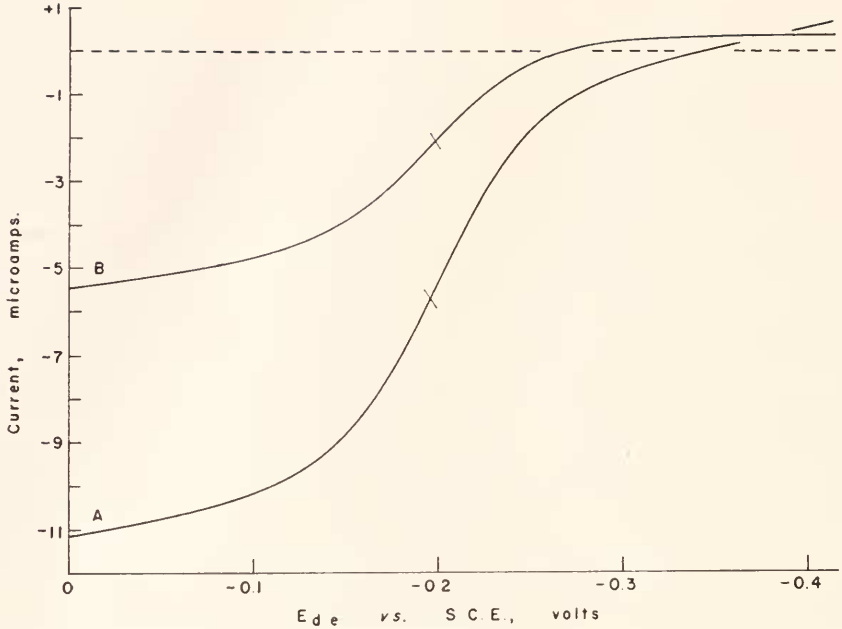

FIGURE 1. Polarograms of reduced glutathione: (A) 1.4×10^{-3} *M* GSH in 0.1 *M* KCl, (B) 7.2×10^{-4} *M* GSH in a complex salt medium also containing yeast, blood serum and methylcellulose (sensitivity X 5; pH 6.6).

were L-cysteine, adrenochrome semicarbazone, ascorbic acid, sodium anthraquinone sulfonate, acetylcholine chloride, riboflavin, and sodium thioglycollate.

A 1.4×10^{-3} *M* solution of reduced glutathione (GSH) in a supporting electrolyte of 0.1 *M* KCl at pH 6.6 produced a polarogram (Curve A, Fig. 1) closely resembling that of Curve A, Figure 2. Addition of 0.005 gm. GSH to a gut content sample increased the wave height without significant change in the previously recorded half-wave potential.

The comparative method of wave height-concentration plots indicated 7.2×10^{-4} *M* GSH in an isotonic medium of mixed salts, nutrients, and methyl cellulose results in a polarogram (Curve B, Fig. 1) almost identical to that recorded from the natural gut content (Curve A, Fig. 2). The Ilkovič equation, mentioned earlier, was not employed in the calculation of GSH concentration. Application of this equation would require a viscosity reading for hind-gut content, and this has not yet been determined.

B. Chemical

GSH produces a violet color in the presence of sodium nitroprusside. A positive reaction occurs when a thin layer of dark brown *Cryptocercus* gut content is tested on a white porcelain surface. Although ascorbic acid does not react with nitroprusside, cysteine and possibly other sulfhydryl compounds effect the positive reaction (Patterson and Lazarow, 1953).

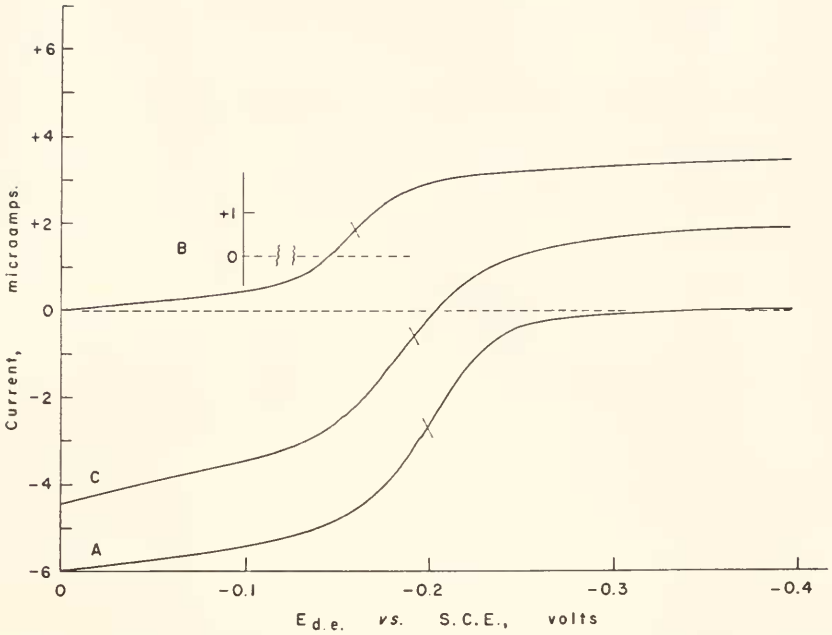

FIGURE 2. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following air passage for one hour, and (C) following nitrogen gas flow, 20 minutes (sensitivity X 5; pH approximately 6.8 at start).

Patterson, Lazarow and Levey (1949) report alloxan in excess reacts with the sulhydryl of GSH forming an unknown substance with an absorption spectrum maximum at 305 mμ. By this method 3×10^{-4} *M* GSH has been identified in hind-gut content cleared of cellulose and protozoa. The authors mentioned also report equivalent molecular amounts of cysteine, cysteinylglycine, glutamylcysteine, and ascorbic acid in the presence of GSH lower the value of GSH determination.

C. Bioassay

Loomis (1955a, 1955b) showed that a concentration of 10^{-5} *M* GSH elicits a characteristic feeding response in starved *Hydra littoralis*. A 1:3000 dilution of *Cryptocercus* gut content induced a strong response, indicating a concentration of GSH somewhere in the order of 10^{-3} *M*. This bioassay, however, is no longer specific for GSH. Lenhoff and Bovaird (1960) obtained the same response with trypsin, and Cliffe and Waley (1958) obtained it with ophthalmic acid.

3. *The apparent source of GSH*

Since variables involved with polarograph operation were held constant, slight variations in GSH concentration, observed in fresh unaltered samples, must be inherent in sample fluids. The ratio between number of cells and surrounding fluid volume probably varies between hosts. If GSH is protozoan and/or bacterial in

origin, it could fluctuate with the rise and fall of these populations. If GSH is a secretion of the host tissues, its concentration could vary in direct proportion with the mass of the insect. The question of primary concern, however, was the source of GSH.

After squeezing, sufficient protozoa remain within the roach to repopulate all species back to normal level within four to six weeks. During this period, contrary to conditions in unsqueezed roaches, many cells appear in various stages of mitosis. Protozoan repopulation is a relatively slow process, but the fluid content is rapidly reestablished. An adequate volume of fluid can be removed from the same roach 24 hours after squeezing for polarographic study.

Polarograms were made at 24 hours, and at the end of the first, third, and fourth weeks during the repopulation period with two groups of squeezed roaches. Results indicate GSH concentration increases directly proportional to the increasing protozoan population.

For the present, the relatively crude method used in estimating repopulation rate during the four-six-week period appears adequate. Three stages in the process were easily recognized. (1) That time immediately following squeezing when the gut fluid contains a few random, scattered, residual cells. (2) A mid-stage follows when fluid contains a large number of cells, but also cell-free areas. (3) Maximum population is reached when a mass of cells appears, each in contact at many points with other cells.

An estimate of one week for total bacterial repopulation, following squeezing, exceeds the mean reproductive potential of such organisms. If host tissue was responsible for GSH secretion, a maximum concentration would be expected (polarographic inspection) within one week after squeezing. Usually three weeks were required before GSH concentration reached 50% of normal. This corresponds to a protozoan population judged to be at the approximate refaunation half-way mark.

Several protozoan species are intimately associated with one or more species of bacteria. The cell membrane of *Barbulanympha* shows uniform cellular arrangement for one species of bacterial rod. The association is permanent. This example of *subordinate* symbiosis, and other associations of a similar nature, prevent final decision regarding the exact source of GSH—the protozoa *per se,* intimate bacterial associates, or both. It is, however, significant to know GSH is not host elicited.

4. *The effect of oxygen and nitrogen on GSH*

The relationship between absence of a polarographic oxygen wave, following exposure of gut content to air or pure oxygen, and GSH concentration must be considered.

A sample of hind-gut content bubbled with air at a slow rate for as long as one hour in the H-cell solution chamber failed to produce an oxygen wave. GSH concentration, however, decreased approximately one-half from a reading of approximately 5.4 μa to 2.5 μa shown in a comparison of Curves A and B, Figure 2. The reduction potential also was shifted 0.04 volt more positive.

Roughly 90% of the initial GSH concentration (presumably oxidized) was reduced in the gut content during nitrogen passage for one hour. Recovery was indicated by an increase in the height of the wave (Curve C, Fig. 2) and a reduction

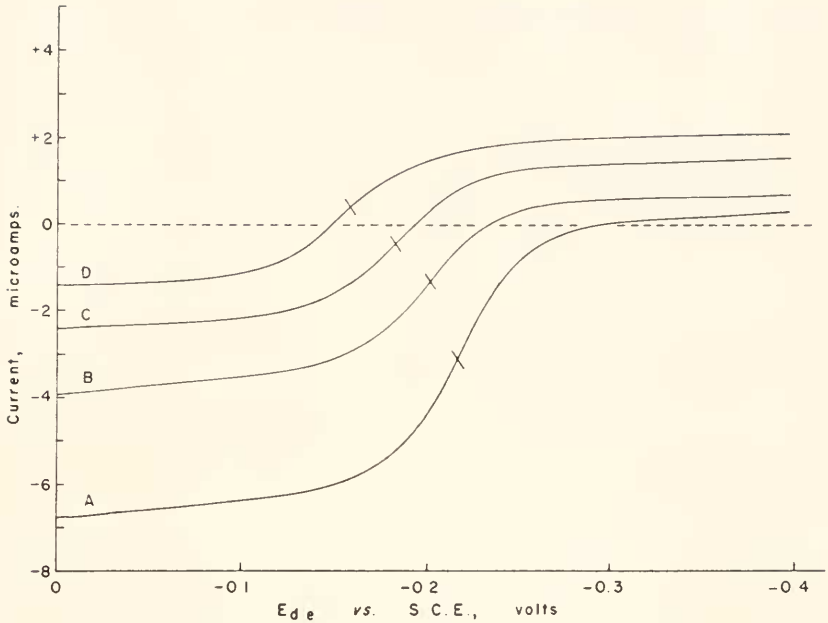

FIGURE 3. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following pure oxygen passage ten minutes, (C) following a 20-minute additional period of pure oxygen passage, and (D) nitrogen gas passage, 40 minutes (sensitivity X 5; pH approximately 6.8 at start).

potential shifted back to a more negative reading. Shift of $-E\ ½$ to a more negative position was first viewed as a result of alkalinity increase following a CO_2 *blow-off*. But, upon further consideration, oxygen passage would also be expected to achieve the same effect, and does not. A change in pH, nevertheless, first in one direction and then the other, appears responsible for corresponding shifts in reduction potential.

Curves B and C, Figure 3, show a progressive reduction in GSH concentration for one sample following contact with pure oxygen. Curve A represents the sample's GSH level prior to oxygen exposure. Curve A differs from the previous experiment's unexposed sample (Curve A, Fig. 2), registering approximately 0.7 μa more GSH and a slightly higher pH, judging from the more negative E ½ position.

Curve B, Figure 3, was recorded following oxygen passage for ten minutes. From a comparison of microampere readings between Curve A (original concentration) and Curve B, estimated GSH drop was 50%.

Curve C, Figure 3, was recorded following oxygen passage lasting 20 additional minutes, in which the GSH level was further reduced. Even under these extreme conditions, in a sample less than 0.5 ml., it was not possible to demonstrate the presence of dissolved oxygen.

Continuing with the same sample, nitrogen passage—following oxygen exposure of the extreme intensity just described—failed unaccountably to demonstrate the

reversibility achieved with nitrogen after air exposure in the earlier experiment (Curve C, Fig. 2). Even forty minutes' contact with nitrogen (Curve D, Fig. 3) did not bring about a curve height increase beyond that recorded in Curve C, Figure 3. The height of Curve D, Figure 3, was depressed even further. In addition, again contrary to results obtained when the air-treated sample was bubbled with nitrogen, a comparison of half-wave potentials in Figure 3 illustrates nitrogen passage was accompanied by a reduction potential shift to a more positive reading.

The phenomena just described are based on repeated observations. Polarograms illustrated trace events related to two treated gut samples; one sample using air passage followed by nitrogen gas (results verified by fourteen experiments), and one sample using "prepurified" oxygen followed by nitrogen gas (results verified by seven experiments).

Although it has not been determined how much oxygen a small sample of gut fluid can *absorb* or *bind* without losing its potential with respect to GSH reduction during nitrogen passage, experiments suggest oxygen can overwhelm the glutathione recovery system.

A. Evidence suggesting enzymatic reduction of GSH

That partial GSH reduction can occur during nitrogen passage through a gut sample under prolonged exposure to air suggests strongly the presence of an appro-

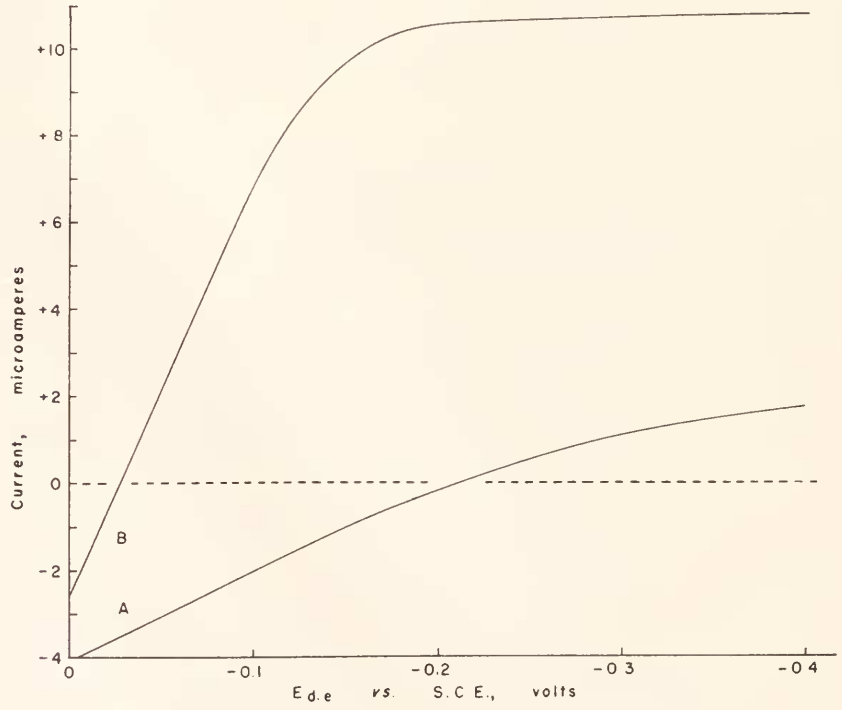

FIGURE 4. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) heated ten minutes, and (B) following pure oxygen passage five minutes (sensitivity X 2).

priate enzyme system. Further evidence favoring the enzyme hypothesis appears when partial glutathione recovery, to the reduced state following air exposure, cannot be achieved in a sample altered by heating over a water bath for 10 minutes at 80° C. Even negligible atmosphere exposure encountered in transferring a heat-treated sample to the electrolysis vessel is sufficient to oxidize GSH, as indicated by disappearance of the reduction wave (Curve A, Fig. 4). Five minutes of oxygen passage then results in such marked saturation of the sample that a prominent oxygen wave (Curve B, Fig. 4) can be recorded.

Glutathione reductase and dehydrogenase assays have not yet been undertaken. Such studies will be resumed following application of present findings to protozoan culture. It is entirely possible that more than one enzyme will be found present in the hind-gut content, accounting for rapid glutathione auto-reduction demonstrated prior to heat alteration.

5. *Evidence suggesting a chemical relationship between glutathione and carbon dioxide*

Polarographic experiments and related observations repeatedly point out another phenomenon in the gut content which might support M. Calvin's (1953) hypothesis for addition of mercaptans across C = O in CO_2 in slightly alkaline media to form S-alkyl monothiocarbonate. He predicted an acid medium could reverse the reaction to release CO_2 and restore GSH:

$$GSH + \overset{O}{\overset{\|}{C}} = O + OH^- \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} R - S - \overset{O}{\overset{\|}{C}} - O^- + H_2O$$

A dense resident population of microorganisms in the hind-gut of *Cryptocercus* ferments ingested cellulose. The fermentation products would be expected to be similar to those reported by Hungate (1939) for *Zootermopsis*—CO_2, hydrogen, and predominantly acetic acid. Complete acid analysis has not been made, but total CO_2 in freshly removed gut content measures 0.12 volume per cent by Scholander's (1947) method.

Fermentation probably continues briefly, following sample introduction into a polarographic vessel (due to continued cell activity), which partially accounts for increase in acidity. Twenty to 30 minutes later the pH drops from the 6.6–7.2 range to 6.4. Within five hours the pH drops to 4.8–5.1, and at 18 hours 4.4. This could explain the shift of half-wave potential in polarograms (Curves A, B, and C, Fig. 5) from −0.196 volt *vs.* S.C.E. made immediately to a more positive potential approximating −0.133 volt after 17 hours.

The protozoa are dead, most are deformed, and some are lysed when the pH reaches 6.4. Motile bacteria are no longer active. The remaining bacterial species constitute a very small number of cells in what was an overwhelmingly populated milieu. These bacterial species can be cultured in a cellulose-nutrient medium which supports growth and reproduction of five protozoan genera normally associated with the bacteria (Ritter, 1959). Bacterial metabolites produced in this culture medium in the absence of protozoa cause the pH to increase, not decrease.

Bacterial influence in this phenomenon of acidification seems to be completely ruled out following observation of toluene-treated gut content samples. Toluene addition to the extent of 20% final dilution failed to alter the pattern of pH decrease to the 4.0–5.0 range within 18 hours.

The cause of pH drop from 6.4 to 4.4 in 18 hours must be accounted for if, as seems to be the case, protozoa and bacteria are not responsible. It appears that bound CO_2 is released in sufficient quantity to cause this pH drop. CO_2 was identified as a major gas constituent, accumulating after several hours as a bubble mound at the interface of a large gut fluid sample layered with heavy mineral oil.

Correlated with this rapid accumulation of CO_2 in the absence of fermentation, polarograms (Curves A and C, Fig. 5) lend additional support to Calvin's hypothesis. As the pH registers progressively lower, GSH concentration increases. From comparative wave height measurement of polarograms A and C, GSH (which almost doubles in quantity within five hours) increases nearly four times the original amount within 17 hours.

This marked wave height increase could indicate mercaptide formation. It is generally accepted that a typical mercaptan characteristic is reaction with heavy metals such as mercury, and with organic compounds, to form mercaptides. And, apparently, there is every opportunity for this reaction to occur in a sample which rests on mercury in the electrolysis vessel.

The mercaptide phenomenon, if it occurs in this gut fluid, does not appear polarographically. The wave height increase represents GSH concentration in-

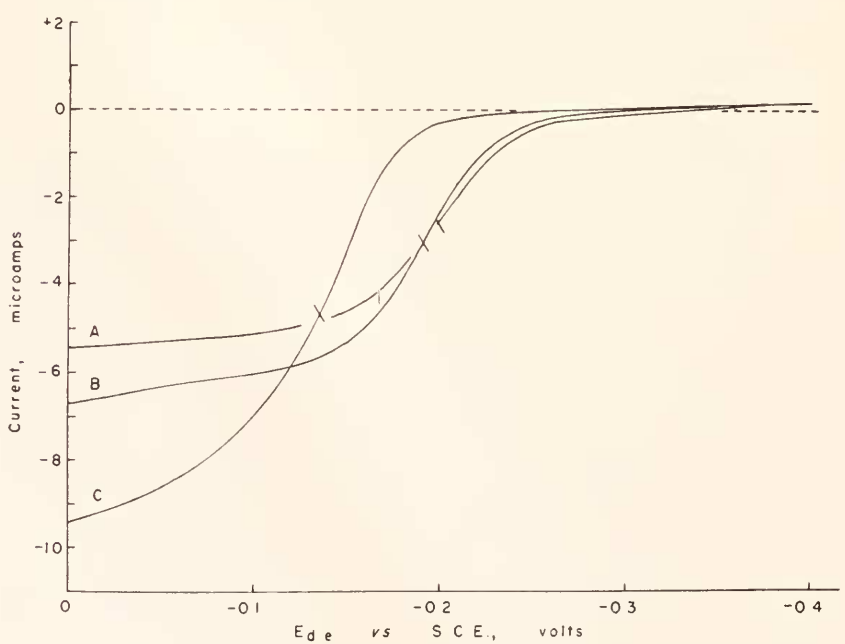

FIGURE 5. Polarograms of the same hind-gut sample from *Cryptocercus*, untreated, and recorded at intervals: (A) immediately, (B) at 20 minutes, and (C) at 17 hours (sensitivity X 5 Curves A and B; X 10 Curve C; pH approximately 6.8 at start; 4.4 at end).

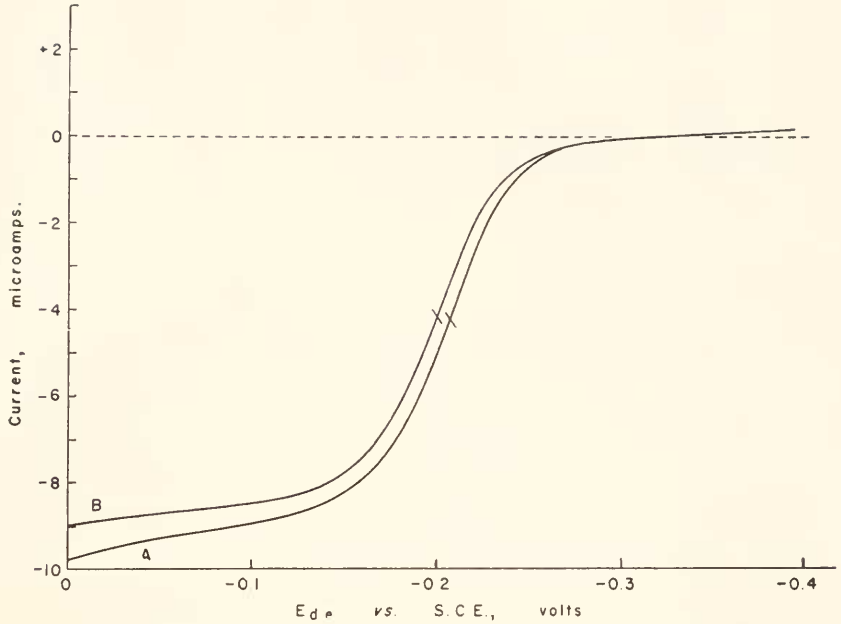

FIGURE 6. Polarograms of two different hind-gut samples from *Cryptocercus:* (A) *aged* 5 hours in the electrolysis vessel and in contact with the mercury pool, pH 5.1, and (B) *aged* 4½ hours in a vessel without mercury contact until transfer to the electrolysis vessel, pH 4.9 (sensitivity X 5).

crease, and the shift in $-E\ ½$ to a more positive reduction potential correlates with pH drop. Evidence for this conclusion rests on:

1. A fluid sample *aged* between four and five hours in a mercury-free container, after removal from the roach and deposit inside the electrolysis vessel, registers a wave (Curve B, Fig. 6) coresponding closely with a fluid sample *aged* the same period in solution chamber contact with mercury (Curve A, Fig. 6).

2. According to Benesch and Benesch (1952), more energy is required for mercaptide complex reduction than for the free compound that gave rise to the complex. Therefore, the resulting reduction wave would register at a more negative E ½ for the mercaptide—not more positive as recorded in this study—to reflect the low dissociation of the Hg-S bond.

3. Also in the event of mercaptide formation, two separate waves would be expected to appear polarographically. The difference between the $-E\ ½$ of the gradually forming mercaptide and the more negative E ½ of disappearing GSH would likely be great enough to initiate two separate reduction waves separated by a shifting plateau.

DISCUSSION

The appreciable presence of atmospheric oxygen coupled with diverse, unique, and efficient animal systems that transport oxygen suggest unspecified claims of anaerobiosis (as to degree) should be questioned. Living membranes present an

ineffectual barrier to oxygen. For that matter, full appreciation of oxygen permeability through synthetic membranes cannot be realized until experience is acquired with a *working* chamber from which all oxygen is meant to be excluded. In other words, at least a minute amount of oxygen probably reaches an organism's most remote anatomical niche. If symbionts dwelling in such a site require an absolute oxygen-free environment, either they or the surrounding host cells must chemically maintain it.

1. *Absence of molecular oxygen in* Cryptocercus *hind-gut content*

A. Direct evidence

The entire problem of determining environmental pO_2 at lower limits is hampered by inadequate indicators and mechanical devices for measurement. Polarography, in spite of extreme sensitivity for detecting oxygen in solution, is incapable of registering ultra-trace amounts. Failure to obtain typical reduction waves for oxygen in *Cryptocercus* hind-gut fluid polarograms is of great analytical significance. However, the question of degree in anaerobiosis still remains unanswered. To report that this environment is free of oxygen to the extent of 10^{-6} *M* is informative, though admittedly inadequate, for culture investigation. Technically, therefore, the term anaerobic should not be used merely on the basis of this information.

B. Indirect evidence

The polarograph also indicated concentration decrease of an unknown substance when gut content was exposed to pure oxygen. Prolonged oxygen exposure failed to leave enough molecular oxygen in solution for polarograph detection. In addition to indicating a substance is exploited in proportion to the amount of oxygen passing into gut fluid, the observation strongly suggests chemical affinity between oxygen and this unknown substance.

Oxygen most certainly diffuses into the lumen from the gut epithelium under natural conditions. Conceivably the oxygen is instantly bound molecularly. Interpretation of these phenomena, based on direct and indirect evidence obtained in polarograms, suggests *Cryptocercus* protozoa live in an anaerobic environment—anaerobic to the extent of maximum physical and chemical maintenance.

2. *Reduced glutathione in* Cryptocercus *hind-gut content*

A. Methodology in identification

Identification of the substance, which registers so prominently on polarograms and appears to function as a strong reducing agent, was difficult. Analyses offering unequivocal interpretation are rare when attempting to single out one organic constituent in the complexity of a biological medium.

Investigative methods included comparative polarography, the classic sodium nitroprusside test, the alloxan "305" method, and the Loomis bioassay technique.

Comparative polarography is a highly accurate procedure in which closely controlled physical conditions are imposed to measure known and unknown solutions. A more extensive survey of potential reducing agents appeared unnecessary because of the method's relative specificity. A known GSH solution produces

a polarogram of nearly identical structure to gut content polarograms. The supporting electrolyte of mixed salts used in the GSH medium was determined by flame photometry of the gut fluid lacking major suspended elements. Viscosity was approximated with methyl cellulose, and the pH of 6.8 required no adjustment. Variation in any one of these—especially pH—caused an expected fluctuation in the $-E\ ½$, but the polarographic curve remained essentially unchanged.

As mentioned earlier, the standard addition method of polarography confirmed the presence of glutathione. Addition of GSH to the unknown sample increased the wave height (concentration) without changing other aspects of the curve.

Following preliminary evaluation of other available methods of GSH analysis for application to this study, one-dimensional paper chromatography—of use after blocking the sulfhydryl group with N-ethylmaleimide (Hanes, Hird and Isherwood, 1950)—indicated greatest specificity. Inadequate sensitivity of the chromatographic method, using GSH concentration determined polarographically as reference, eliminated chromatographic application.

The alloxan "305" method functioned as a qualitative test only. GSH concentration determined in the supernatant portion of centrifuged gut content was less than half the amount demonstrated polarographically. Although substances already mentioned compete with GSH in alloxan reaction, it is possible only two need be considered. Cysteine and ascorbic acid, though potentially reactive at the dropping mercury electrode, do not appear polarographically. Glutamylcysteine and/or cysteinylglycine might be involved in competitive absorption. Awaiting further investigation, one might guess that γ glutamylcysteine, an intermediate in GSH synthesis probably to the exclusion of cysteinylglycine (Snoke and Bloch, 1952), is of more vital concern in evaluating potential alloxan competition with GSH.

The nitroprusside method and the bioassay technique using *Hydra* lack specificity in GSH identification. Nevertheless, nitroprusside identified the substance as a sulfhydryl compound, and *Hydra* gives a feeding response only in the presence of reduced glutathione. Trypsin, as yet unidentified in *Cryptocercus,* has not been ruled out as an alternative stimulant for *Hydra.*

This represents by no means an exhaustive methodology for assaying biologically-occurring glutathione. Each chemical test available is nonspecific or limited to some degree. General disagreement usually exists regarding the number of chemical tests necessary before identification is scientifically acceptable. In this polarographic study, however, more than mere substantial evidence accumulated to support glutathione assessment. Results derived from both nitroprusside and *Hydra* assays were conclusive yet predicated upon far less specific analysis.

On the strength of collective information afforded by these tests, along with confirming opinions of Professors K. E. Bloch, J. T. Edsall, and A. M. Pappenheimer, GSH is a constituent of *Cryptocercus* hind-gut content. GSH is responsible for maintenance of the oxidation-reduction potential observed polarographically.

B. The site of synthesis

Since more than 30 species of protozoa inhabit *Cryptocercus,* it must still be determined which of these are involved in GSH synthesis. The problem is further complicated by intimate, and, in some cases, permanent, bacterial association with the cell membrane of certain protozoan species. Evidence suggests protozoa are the site for this synthesis, but not necessarily the sole metabolic source of GSH.

C. Oxidation-reduction activity

It was impossible to assess fully results obtained in experiments in which gas was directed through freshly obtained gut content samples. It was clear that oxygen decreased the concentration of reduced glutathione. This was interpreted as an oxidative phenomenon in which GSH was converted to the disulfide form (GSSG). Although nitrogen passage was capable of partially restoring the glutathione wave height in air-exposed samples, the precise mechanism was not discernible. It has been assumed temporarily that this wave extension represents a reduction of GSSG to GSH—the nitrogen gas being most indirectly involved.

Wieland (1953) stated reduction of GSSG can be accomplished with various reducing agents, other mercaptans in excess, nascent hydrogen, Na or Li in NH_3, and by other substances. He also pointed out enzymatic reduction is frequently responsible. Whatever the cause, a point exists—polarographically demonstrated—at which nitrogen can no longer carry out its indirect role. This occurs following gut content exposure to pure oxygen in excess of ten minutes. Glutathione oxidized to this extent could not be *reactivated* as could samples exposed to lesser amounts of oxygen.

Opinions sought are totally inadequate in accounting for contradictory evidence concerning the effect of nitrogen following varied aerations of gut content—air *versus* pure oxygen. Interpretation of present phenomena obtained polarographically must await correlation with results from additional analyses now contemplated.

Since the oxidation-reduction potential of gut content can also be rendered inactive by heat, the tentative conclusion for the presence of at least one enzyme system seems justified. Glutathione reductase, according to Vennesland and Conn (1953), is the thermolabile enzyme responsible for the reduction of GSSG by tissues. GSSG is reactive with TPN-reducing systems. Accordingly: $TPNH + GSSG + H^+ \rightarrow TPN^+ + 2GSH$

D. A proposed role in pCO_2 and pH regulation

Culture studies in progress demonstrate glutathione can now be assigned an extracellular role in anaerobic maintenance. The importance of this mercaptan in growth and other physiological activities is well substantiated, but whether it can be assigned an additional function in the gut of *Cryptocercus* is still problematical.

Speculation regarding GSH capacity to combine with CO_2 to form an alkyl compound, as proposed by Calvin, is a tempting hypothesis. Culture studies of the protozoa definitely show a very narrow range of pCO_2 tolerance. Experimentally, membrane selectivity can be altered dramatically when cells are exposed to an excess of this gas, but protozoa move directly to a higher pCO_2 gradient when placed in a sub-optimal region of the culture medium.

Hind-gut protozoa, therefore, engaged in active fermentation yet highly sensitive to an altered pCO_2 threshold, would have marked survival potential if a mechanism were available for chemical regulation of this metabolite. Theoretically GSH could function as a pH-regulated pCO_2 buffer, supplementary to more direct pCO_2 control by the roach's external respiratory apparatus. Conceivably, even though pH might *trigger* this mechanism to reestablish an equilibrium between S-alkyl monothiocarbonate and GSH, pH, in turn, could be regulated, in part at least, by the mechanism. Certainly the host roach and its protozoa are confronted

by environmental factors that challenge optimal metabolism, such as conditions imposed during molting and winter.

I am very much indebted to Dr. L. R. Cleveland for invitation to work at the Biological Laboratories, for his advice, and for his criticism of the manuscript. I am grateful for the assistance of Dr. W. F. Loomis, and for advice and suggestions regarding polarography and the interpretation of polarograms from Professor J. J. Lingane and Dr. Allen J. Bard. Professors Bloch, Edsall, Pappenheimer, and Wald were consulted during various phases of this study. Their interest and counsel is greatly appreciated.

Summary

1. Polarography, employing a mercury-dropping electrode and an electrolysis vessel for analysis of sample slightly less than 0.1 ml., was applied in this study.

2. Polarograms of *Cryptocercus* hind-gut content lack an oxygen wave and indicate absence of dissolved oxygen to the extent of apparatus sensitivity (10^{-6} *M*).

3. A single polarographic wave registers a half-wave potential approximating −0.2 volt *vs.* S.C.E. within a 0- to −0.4-volt span. Specificity of this method enabled identification of reduced glutathione (7.2×10^{-4} *M*) following comparative inspection of numerous reducing agents. Supplementary assays using sodium nitroprusside, alloxan monohydrate, and *Hydra* confirmed the presence of reduced glutathione in hind-gut content.

4. Flagellate protozoa, symbionts in the hind-gut fluid of *Cryptocercus,* represent the site of glutathione synthesis.

5. Prolonged oxygen passage through hind-gut samples (0.1–0.5 ml.) fails to alter polarograms with respect to negative pO_2 registration, but reduced glutathione concentration is decreased in direct proportion to oxygen exposure intensity.

6. Partial restoration of reduced glutathione concentration following nitrogen gas passage through samples moderately exposed to oxygen cannot be explained.

7. Brief atmosphere contact with heat-altered hind-gut samples results in polarograms showing pO_2 contamination and an absence of reduced glutathione. Until enzyme analyses are completed, sample alteration by heat merely suggests presence of a mechanism for glutathione maintenance in the reduced state.

8. *Cryptocercus* hind-gut content is anaerobic to the extent of maximal physical and chemical maintenance as are the microorganisms normally living within it. Reduced glutathione is considered of major importance in this role.

9. Some evidence exists which may support Calvin's hypothesis for addition of mercaptans across carbonyls in a biological sample. The value of such a mechanism to *Cryptocercus* and its symbionts is theorized.

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